Biosynthesis of UDP-glucuronic acid in developing soybean embryos: possible role of UDP-sugar pyrophosphorylase

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During soybean [*Glycine max* (L.) Merrill] embryo development, cell wall polysaccharides (CWPs) derived from UDP-glucuronic acid (UDP-GlcA) (uronic acids, arabinose, xylose) exhibited a linear increase during the period of 25–45 days after flowering (daf). At embryo maturity, CWPs derived from UDP-GlcA accounted for 39% of total CWPs. To ascertain the relative importance of the nucleotide sugar oxidation (NSO) and the *myo*-inositol oxidation (MIO) pathways to UDP-GlcA biosynthesis, UDP-glucose (UDP-Glc) dehydrogenase (UDP-Glc DH, EC 1.1.1.22) and UDP-glucuronic acid pyrophosphorylase (UDP-GlcA PPase, EC 2.7.7.44) activities, respectively, were measured in desalted extracts of developing embryos. UDP-Glc DH and UDP-GlcA PPase activities, expressed on a per seed basis, increased 3.5- and 3.9-fold, respectively, during the period of 25–45 daf. However, UDP-GlcA PPase activity was 35–50-fold greater than UDP-Glc DH activity. The soybean UDP-sugar pyrophosphorylase gene (*USP1*), a homolog of pea *USP*, and a candidate gene for UDP-GlcA PPase, was cloned and the recombinant enzyme characterized. Recombinant soybean USP1 (71 kDa) exhibited high activity with glucuronic acid 1-phosphate (GlcA-1-P), glucose 1-phosphate (Glc-1-P) and galactose 1-phosphate (Gal-1-P), but low activity with mannose 1-phosphate (Man-1-P), N-acetylglucosamine 1-phosphate and Glc-6-P. Determination of kinetic constants indicated that USP1 has a higher affinity for GlcA-1-P ($K_m = 0.14 \pm 0.02$ mM) than for Glc-1-P ($K_m = 0.23 \pm 0.02$ mM). Semiquantitative RT-PCR was used to measure transcript levels of the UDP-glucose DH (*UGD*) and *USP* gene families in developing soybean embryos. Transcript levels, normalized to the 18S rRNA controls, were greater for *UGD* than *USP* throughout embryo development. The possibility that USP serves as UDP-GlcA PPase, the terminal enzyme of the MIO pathway, is discussed.

Abbreviations – Ara, arabinose; BSA, bovine serum albumin; CWP, cell wall polysaccharide; daf, days after flowering; Fuc, fucose; Gal, galactose; Gal-1-P, galactose 1-phosphate; Glc, glucose; Glc-1-P, glucose 1-phosphate; Glc-6-P, glucose 6-phosphate; GlcA, glucuronic acid; GlcA-1-P, glucuronic acid 1-phosphate; Man, mannose; Man-1-P, mannose 1-phosphate; MIO, *myo*-inositol oxidation; NSO, nucleotide sugar oxidation; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; UDP, uridine diphosphate; UDP-Ara, UDP-arabinose; UDP-Glc, UDP-glucose; UDP-Xyl, UDP-xylose; UDP-GlcA, UDP-glucuronic acid; USP, UDP-sugar pyrophosphorylase; UDP-Glc DH, UDP-glucose dehydrogenase; UDP-GlcA PPase, UDP-glucuronic acid pyrophosphorylase; Xyl, xylose.

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Introduction

Soybean [*Glycine max* (L.) Merr.] seeds are a valuable source of protein and oil used for both human and livestock nutrition. Depending on genotype and environment, soybean seeds consist of approximately 20% oil, 40% protein and 18% cell wall polysaccharides (CWPs) on a dry weight basis (Hartwig and Kilen 1991, Stombaugh et al. 2000, Wilson 2004). The benefits of increasing the oil and/or protein content of soybean seeds have been previously described (Cober and Voldeng 2000, Wilson 2004, Panthee et al. 2005). One approach to achieving this goal involves shifting carbon allocation in developing embryos from CWPs to oil or protein. Matrix polysaccharides (pectin, hemicellulose) are a significant component of CWPs in soybean seeds (Stombaugh et al. 2000, 2003). Previous research has indicated that significant differences in the matrix polysaccharide content of cell walls of soybean seeds exist, and that a genetic approach involving the redirection of carbon allocation from CWPs may yield seeds with increased oil or protein content (Stombaugh et al. 2000, 2003). However, pursuing this approach requires a better understanding of matrix polysaccharide biosynthesis in developing soybean seeds.

For over 25 years, it has been known that plants have two pathways for the synthesis of UDP-glucuronic acid (UDP-GlcA), a key precursor for the formation of matrix polysaccharides (Feingold and Avigad 1980, Loewus and Loewus 1983, Kärkönen 2005) (Fig. 1). The nucleotide sugar oxidation (NSO) pathway involves UDP-glucose dehydrogenase (UDP-Glc DH) (EC 1.1.1.22), which catalyzes the irreversible conversion of UDP-glucose (UDP-Glc) to UDP-GlcA. Purified enzyme has been characterized in soybean (Stewart and Copeland 1998, Stewart and Copeland 1999, Hinterberg et al. 2002) and sugarcane (*Saccharum* spp. hybrid) (Turner and Botha 2002). UDP-Glc DH has a subunit molecular mass of 50–55 kDa and exhibits strong feedback regulation by UDP-xylose (UDP-Xyl) (Steward and Copeland 1998, 1999, Hinterberg et al. 2002, Turner and Botha 2002). In most plant species examined to date, it appears that the genes encoding UDP-Glc DH comprise a small gene family (*UGD*), with four genes in *Arabidopsis* (Reiter and Vanzin 2001, Seifert 2004), two genes in tobacco (*Nicotiana tabacum*) (Bindschedler et al. 2005), three genes in poplar (*Populus tremula* × *tremuloides*) (Hertzberg et al. 2001), and at least two genes in maize (*Zea maize* L.) (Kärkönen et al. 2005). In contrast, soybean has been reported to have a single *UGD* gene (Tenhaken and Thulke 1996). Measurement of *UGD* transcript abundance in soybean indicated high expression in roots, the vascular system and young, meristematic regions (Tenhaken and Thulke 1996). The expression pattern for *UGD1* in *Arabidopsis* has been analyzed using promoter–reporter gene fusions (Seitz et al. 2000). As found in soybean, *UGD* was highly expressed in roots and vascular tissues. In poplar, *UGD* was expressed primarily in developing xylem and young leaves and was induced by sucrose and osmoticum (Johansson et al. 2002). Overall, the evidence suggests that *UGD* is a small gene family in most plant species and that expression of *UGD* genes is tissue dependent and developmentally regulated.

The second pathway for the biosynthesis of UDP-GlcA, the *myo*-inositol oxidation (MIO) pathway, involves three enzymatic steps catalyzed consecutively by: *myo*-inositol oxygenase (EC 1.13.99.1); glucuronokinase (EC 2.7.1.43); and UDP-glucuronic acid pyrophosphatase (UDP-GlcA PPase) (EC 2.7.7.44, glucuronate-1-phosphate uridylyltransferase) (Loewus and Loewus 1983) (Fig. 1). *Myo*-inositol oxygenase catalyzes an irreversible reaction and is believed to play an important role in regulating carbohydrate flux through the pathway (Kanter et al. 2005). UDP-GlcA PPase, the terminal enzyme of the pathway, is unique to plants (Loewus and Loewus 1983) and has been partially characterized in semipurified fractions (Feingold et al. 1958, Roberts 1971, Roberts and Cetorelli 1973, Dickinson et al. 1977, Hondo et al. 1983). The substrate specificity and molecular mass of UDP-GlcA PPase have not been clearly defined. The enzyme measured in barley seedlings did not exhibit cross-reactivity with glucose 1-phosphate (Glc-1-P) (Roberts 1971). An enzyme fraction isolated from mung bean (*Phaseolus aureus*) exhibited activity with both glucuronic acid 1-phosphate (GlcA-1-P) and GalA-1-P (Feingold et al. 1958). Barley
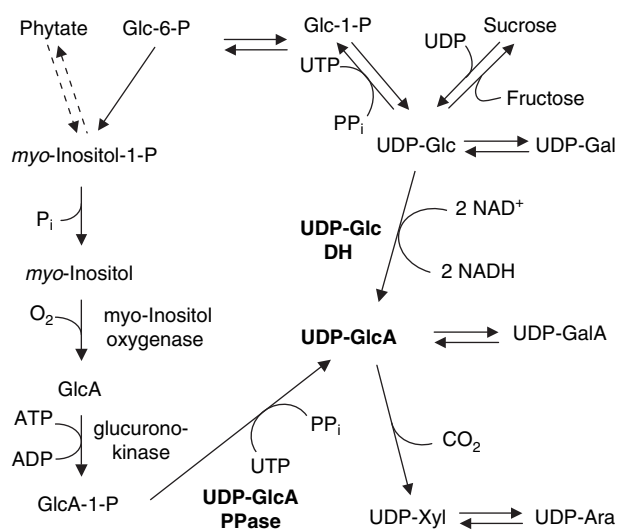

Fig. 1. Nucleotide sugar biosynthesis. UDP-GlcDH, UDP-glucose dehydrogenase; UDP-GlcA, UDP-glucuronic acid; UDP-GlcA PPase, UDP-glucuronic acid pyrophosphorylase. Adapted from Feingold and Avigad (1980).

UDP-GlcA PPase has a molecular mass of approximately 35 kDa (Roberts 1971). However, the estimated molecular mass of UDP-GlcA PPase from common cattail (*Typha latifolia* L.) pollen was approximately 71 kDa (Hondo et al. 1983). The gene for UDP-GlcA PPase has not been identified. However, the gene for UDP-sugar pyrophosphorylase (*USP*), a broad-substrate pyrophosphorylase that exhibits activity with GlcA-1-P, was recently cloned in pea (*Pisum sativum*) (Kotake et al. 2004).

Although both the NSO and MIO pathways can lead to UDP-GlcA synthesis, the relative importance of the two pathways in plants is not clear; it appears to be dependent on tissue and stage of development (Seitz et al. 2000, Kärkönen 2005, Kärkönen et al. 2005). One approach that has been used to evaluate the relative importance of the two pathways involves comparing the activities of UDP-GlcA PPase and UDP-Glc DH, considered to be marker enzymes for the MIO and NSO pathways, respectively (Roberts 1971, Roberts and Cetorelli 1973, Amino et al. 1985, Witt 1992). These investigations have found that the relative activities of the two marker enzymes are tissue dependent. No similar comparisons of the relative importance of the NSO and MIO pathways in the biosynthesis of UDP-GlcA have been made in developing seeds. The objectives of the research described herein were to: (1) characterize the CWP composition of developing soybean embryos; (2) assay UDP-Glc DH and UDP-GlcA PPase, as marker enzymes for the NSO and MIO pathways, respectively, during embryo development; (3) clone and characterize soybean *USP*, a candidate gene for UDP-GlcA PPase; and (4) measure transcript levels for the *UGD* and *USP* gene families in developing soybean embryos.

Materials and methods

Chemicals

Radiolabeled substrates, [^{14}C]UDP-GlcA (313 mCi mmol^{-1}) and [^{14}C]UDP-Glc (303 mCi mmol^{-1}), were purchased from MP Biomedicals (Irvine, CA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Plant material

Soybeans (*Glycine max* L. Merr. cv. 'Lambert') were grown in the field in St Paul, MN in 2004. Flowers on all plants were tagged as they opened. Pods were harvested from randomly chosen plants at 15, 25, 35 and 45 days after flowering (daf) and at maturity. Replicates were taken from different rows and harvested on different dates. Seeds were removed from the pods, counted, weighed, frozen in liquid nitrogen and stored at −70°C. At 15 daf, the entire seed was frozen, but for seeds harvested at 25, 35 and 45 daf, the seed coat was removed from the embryo (cotyledon plus embryonic axis) before freezing.

Seed composition and cell wall polysaccharide analysis

Seeds were lyophilized, weighed, ground to pass through a 1-mm screen, and then incubated at 50°C for 24 h, which allowed a stable moisture content to be attained without altering the chemical composition of the material. Protein was quantified by microkjeldahl (N × 6.25). Oil content was calculated from the difference in mass before and after Soxlet extraction with hexane. CWPs were quantified in starch-free, 80% ethanol-insoluble samples by the Uppsala total dietary fiber method (Theander et al. 1995). Uronic acids were quantified colorimetrically by the method of Ahmed and Labavitch (1977). Soluble sugars (glucose (Glc), fructose, sucrose, raffinose, stachyose) were extracted overnight with 80% ethanol at 60°C. The residue was subsequently treated with α-amylase and amyloglucosidase to hydrolyze starch. Glucose and starch were recovered in 80% ethanol. Soluble sugars and Glc from starch were quantified on a BP-100 H$^+$ carbohydrate column (300 mm × 7.8 mm) with a BP-100 H$^+$ guard column (50 mm × 4.6 mm) (Benson Polymeric Inc., Sparks NV) using an Agilent 1100 HPLC system with quad pump, autosampler, column heater and refractive index detector. Column and detector temperature was 35°C. The mobile phase was degassed, thermally stable, Nanopure water with a flow rate of 0.4 ml min^{-1} and a run time of 45 min (isocratic). The injection volume was 50 μl. Quantification of sugars, based on peak area of standards, was determined by using CHEMSTATION A.08.03 software (Agilent Technologies, Palo Alto, CA).

Cloning and expression of soybean *USP1*

The 5′-end of the soybean *USP1* gene was isolated using the SMART RACE kit (BD Biosciences, Palo Alto, CA) with mRNA isolated from cotyledons harvested at 45 daf. The gene-specific primer 5′-TCGCCGTCAGGGTATCCACTT-GCTCT-3′, derived from soybean EST no. AI736043, gave a single 1.1 kb product. This product was cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. Sequence information from the 5′-RACE PCR product was used to design a 5′ cloning primer. A full-length

soybean USP product was amplified using the SbUSP5′-UTR primer (5′-GCACTTATCCTCATCAAACACCTT-3′) in combination with the SbUSP3′-UTR primer (5′-CGTCTTCTCACCACCACCTTCC-3′) with Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The template was cDNA created from total RNA isolated from soybean leaves.

USP peptide sequence comparison

Pea *USP* (GenBank #AB178642) was used as the query sequence in BLAST searches of GenBank for *Arabidopsis* and of the TIGR EST databases (www.tigr.org) for *Medicago truncatula* and rice (*Oryza sativa*). *Arabidopsis* locus At5g52560, *Medicago* EST contig TC77530 and rice contig TC238241 were identified as the closest *USP* homologs. Soybean *USP* was cloned and sequenced as described above. The open reading frames of these sequences were translated to give predicted peptide sequences, and the sequences were aligned using VectorNTI. The *Medicago truncatula* sequence was edited to remove a frame shift at nucleotide 1021.

Expression and assay of recombinant USP

The coding region of soybean *USP1* was cloned into expression vector pET28b (Novagen, La Jolla, CA). The sequence was fully verified after cloning. The expression plasmid was transformed into *Escherichia coli* strain BL21pLysS for protein production. Cultures were grown in LB medium at 37°C to an optical density of 0.6, and then induced by addition of Isopropyl β-0-thiogalacto-pyranoside to a final concentration of 1 mM. After induction, the cultures were incubated at 22°C for 5 h. Cells were harvested by centrifugation (8000 g, 15 min) and resuspended in 1/20 culture volume of lysis buffer (0.4 M NaCl, 100 mM HEPES, 10 mM imidazole, 5 mM dithiothreitol, pH 7.5) with 2 units ml^{-1} of DNase, and then frozen at − 20°C. USP was purified from the soluble cytoplasmic protein fraction by binding to His-Link affinity resin (Promega), according to the manufacturer's instructions. USP was eluted with a buffer containing 500 mM imidazole, 10 mM HEPES, pH 7.5, and 1 mM DTT. The fraction was then desalted on a PD-10 column (Amersham Biosciences, Piscataway, NJ) equilibrated with buffer A [20% (w/v) sucrose, 50 mM N-tris[Hydroxymethyl]methyl-3amino-propane sulfonic acid, pH 8.0, 1 mM MgCl$_2$, 1 mM DTT]. The poly-His tag was removed by thrombin cleavage for 16 h at 4°C with 1 unit of thrombin per mg of USP. Thrombin was removed by affinity chromatography according to the manufacturer's instructions (Novagen).

The UDP-GlcA PPase activity of recombinant USP1 was measured in both the forward direction [conversion of GlcA-1-P to UDP-GlcA as measured by the production of inorganic pyrophosphate (PP$_i$)] and the reverse direction (conversion of [^{14}C]UDP-GlcA to [^{14}C]GlcA-1-P). For the forward reaction, the assay medium contained 10 mM TAPS, pH 8.0, 2 mM MgCl$_2$, 0.2 units ml^{-1} inorganic pyrophosphatase, 1 mM UTP and 1 mM sugar-phosphate substrate. Added inorganic pyrophosphatase was in excess of that needed to completely hydrolyze PP$_i$ produced in the assay. The reaction was started by the addition of assay medium to tubes containing desalting buffer [20% sucrose (w/v), 50 mM TAPS, pH 8.0, 1 mM MgCl$_2$, and 1 mM DTT] plus recombinant enzyme (50 ng) in a final reaction volume of 0.5 ml. After incubation for 10 min at 30°C, formation of phosphate (P$_i$) was measured by the method of Aoyama et al. (2001). Briefly, an equal volume of 200 mM acetic acid/sodium acetate buffer, pH 4.0, containing 3% (w/v) ammonium molybdate and 0.1 volume of 1% (w/v) ascorbic acid, was added to each tube. Color was allowed to develop for 30 min at room temperature, and absorbance at 720 nm was measured. A standard curve prepared in assay buffer gave a linear reaction over the range 0–250 μM PP$_i$.

UDP-GlcA PPase activity in the reverse direction was determined by measuring the formation of [^{14}C]GlcA-1-P from UDP-[^{14}C]GlcA and PP$_i$ by using a modification of the protocol of Szumilo et al. [1996]. The reaction mixtures contained the following components in a final volume of 50 μl: 100 mM TAPS, pH 8.0, 2 mM MgCl$_2$, 2 mM UDP-[^{14}C]GlcA (0.5 mCi mmol^{-1}), 5 mM sodium pyrophosphate and purified recombinant enzyme. After 10 min of incubation at 30°C, the reactions were terminated by adding 0.5 ml of 5% (w/v) trichloroacetic acid. Nucleotides were adsorbed on charcoal by adding 0.3 ml of ENVI-Carb 120/400 (150 mg ml^{-1} in water) (Sigma-Aldrich). The suspension was shaken for 2 min and the charcoal pelleted by centrifugation (16 000 g, 2 min). The supernatant was removed and saved, and the charcoal was washed with 1.0 ml of water. The supernatants were combined and 1.0 ml was added to 15 ml of EcoLume (MP Biomedicals, Irvine, CA) for scintillation counting. Blank controls were run simultaneously and contained all components with the exception of sodium pyrophosphate. The formation of [^{14}C]GlcA-1-P, in the reverse assay, was verified by separating the reaction products on cellulose TLC plates. The reverse assay was stopped by adding 2 μl of 0.5 M EDTA, and 2 μl of the reaction mixture was applied to a Cellulose 300 flexible-backed TLC plate (Selecto Scientific, Swanee, GA). The plate was developed with n-butanol/acetone/acetic acid/5% ammonium hydroxide/water (3.5 : 2.5 : 1.5 : 1.5 : 1) (Randerath 1962). The standard lanes, containing 20 nmol of either UDP-GlcA or GlcA-1-P, were sprayed with an acidic molybdate spray (1% ammonium molybdate, 3%

perchloric acid and 0.1 M HCl) to detect phosphate esters (Bandurski and Axelrod 1951, Bieleski and Young 1963). The corresponding bands from the reaction lanes were cut out and placed in 7-ml scintillation vials with 0.2 ml of water. After vortexing, 5 ml of EcoLume was added for scintillation counting.

Enzyme kinetics

The non-radioactive forward assay described above was modified for determination of kinetic parameters. Inorganic pyrophosphatase was omitted from the assay medium. After a 10-min incubation with purified soybean USP1 (100 ng) at 30°C, the reaction was stopped by boiling for 2.5 min. The samples were then placed on ice to cool. Inorganic pyrophosphatase [50 μl of 1 unit ml^{-1} in 50% (v/v) glycerol] was added and the samples were incubated for 10 min at 30°C to cleave PP$_i$. Phosphate was measured as described above. K_m and V_{max} for GlcA-1-P, Glc-1-P and UTP were determined by varying the concentration of UTP from 0.025 to 0.5 mM at each of five fixed concentrations of GlcA-1-P or Glc-1-P (0.1–2.0 mM). The data were fitted to an equation describing a two-substrate ping-pong mechanism using non-linear regression software (GRAFIT 3.0, Erithacus Software). K_m and V_{max} for UDP-GlcA and UDP-Glc were determined by varying the concentration of UDP-sugar from 0.1 to 2 mM at a fixed concentration of NaPP$_i$ (1 mM). K_m and V_{max} for PP$_i$ were determined by varying the concentration of PP$_i$ from 0.1 to 1 mM at a fixed concentration (2 mM) of either UDP-GlcA or UDP-Glc. The data were fitted to the Michaelis–Menten equation using non-linear regression software (GRAFIT 3.0). The concentrations of fixed substrates were empirically determined to be saturating.

Embryo extraction and enzyme assays

Preliminary results indicated that different extraction media were required to stabilize UDP-Glc DH and UDP-GlcA PPase activities in crude extracts. Therefore, enzyme activities were determined on separate subsamples of seeds taken from one sampling date and processed separately for the two enzyme assays. For UDP-Glc DH assays, seeds were ground with a mortar and pestle in buffer A [10 mM EDTA, 5 mM DTT, 0.5 mM NAD$^+$, 20% (v/v) glycerol, 40 mM Tris-HCl, pH 7.5] using five volumes of buffer per gram of seeds. All steps were performed at 4°C unless otherwise indicated. The homogenate was filtered through two layers of cheesecloth and one layer of miracloth, and then centrifuged (6000 g, 10 min). Crude extracts were desalted using PD-10 gel filtration columns equilibrated in buffer A.

UDP-Glc DH activity was measured in the presence of 1 mM pyrazole to eliminate the confounding of assay results by alcohol dehydrogenase (Litterer et al. 2005). Activity was determined spectrophotometrically by measuring the formation of NADH at 340 nm at 30°C in a 1-cm light path cuvette. The reaction mixture contained 50 mM Bicine, pH 8.5, 2 mM NAD$^+$, 1 mM UDP-glucose, 1 mM pyrazole and 0.2 ml of desalted soybean extract in a total volume of 1 ml. The reaction was started by the addition of NAD$^+$ and run for 6 min. Activity was measured during the period 1–6 min after reaction initiation. The reaction rate was linear during this interval. Activity was corrected for background reaction measured in the absence of UDP-Glc.

UDP-GlcA PPase was assayed from extracts prepared and desalted in buffer B [1 mM MgCl$_2$, 0.1% Triton X-100 (v/v), 0.5 mM phenylmethylsulfonyl fluoride, 10 mM 2-mercaptoethanol, 500 mM KCl, 50 mM Tris-HCl, pH 8.0]. All steps of the enzyme extraction and desalting were the same as described above for UDP-Glc DH. UDP-GlcA PPase activity in the reverse direction was determined by measuring the formation of [^{14}C]GlcA-1-P from UDP-[^{14}C]GlcA and PP$_i$ using a modified protocol of Szumilo et al. (1996) as described above.

RNA purification and semiquantitative RT-PCR

Total RNA from soybean embryos and plant tissues was purified from samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was treated with DNase at 37°C for 30 min in a 200-μl reaction containing 5 units ml^{-1} RQ1 DNase (Promega), 200 units ml^{-1} RNasin (Promega), 50 mM Tris (pH 7.8), 10 mM MgCl$_2$ and 1 mM DTT. The RNA was extracted from the DNase reaction with a phenol/chloroform extraction and then precipitated with ethanol prior to use in the reverse transcription reaction. Reverse transcription was carried out with 1 μg of total RNA for each sample, using Improm-II reverse transcriptase and random primers (Promega). To compensate for variability in RNA quality, quantification and tube-to-tube preparation between samples, multiplex PCR was used with the 18S ribosomal transcript as the internal control. QuantumRNA 18S Internal Standards (Ambion, Austin, TX) were used along with gene-specific primers in multiplex PCR. All PCR reactions were carried out according to the QuantumRNA 18S Internal Standards protocol provided by the manufacturer. Ratios of 18S primers/18S competimers were tested to select a ratio that gave an 18S PCR product with similar yield in all samples for each gene of interest. A 3 : 7 ratio of 18S primers/18S competimers was used for *USP*, and a 1 : 9 ratio was used for *UGD*. The *UGD* primers were 5'-GATWGCHATTCTGGGRTTTGHTTC-3' and 5'-GG-CATGTCYTTGAGCCATGRR-3', designed with degenerate

bases to amplify an approximately 500-bp product from the UGD homologs in soybean. Primers for *USP* were 5′-CCATAGTGCAACTTCTGGAG-3′ and 5′-GCCTTCAAGTGAAATTTGCC-3′, giving a 465-bp product near the 3′ end of the coding region.

Protein determination

Protein was determined using the Bradford (1976) assay with bovine serum albumin (BSA) as the standard.

Results

Cell wall composition

To place UDP-GlcA synthesis in the context of embryo development, embryo composition was determined at 10-day intervals during the linear growth phase of seed fill (25–45 daf), and during seed maturation (45 daf to maturity) (Fig. 2). During the linear phase of growth, rapid increases in oil, protein and CWPs occurred. Both CWP and protein synthesis continued to increase during seed maturation, but oil accumulation leveled off. During seed maturation, soluble sugars increased and starch decreased by similar amounts. Expressed on a gram per dry weight basis, CWPs accounted for 9.8% of the mature embryo. The predominant monosaccharides in cell walls of developing embryos were galactose (Gal), Glc, arabinose (Ara), and uronic acids (Fig. 3). Cell wall monosaccharides directly derived from UDP-GlcA [uronic acids, Ara, xylose (Xyl)] accumulated steadily during
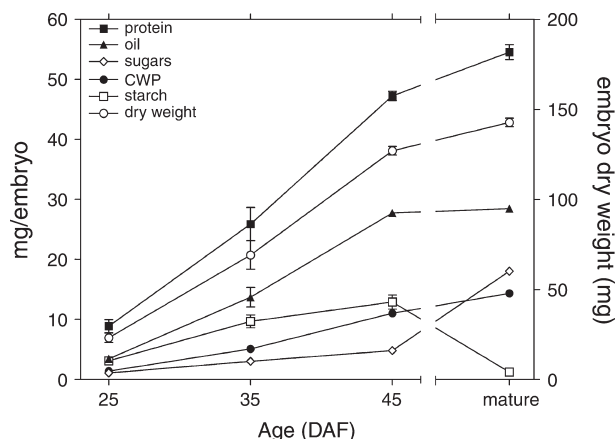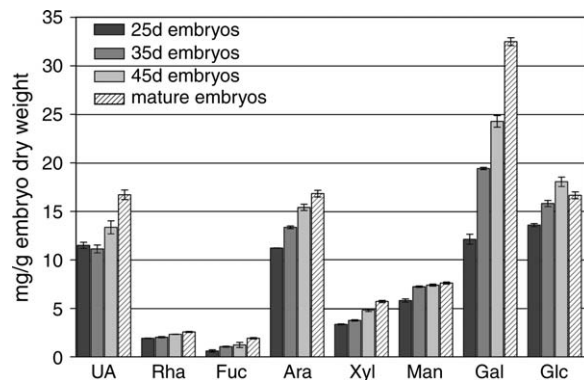
Fig. 3. Cell wall monosaccharides in developing soybean embryos. Monosaccharide analyses were performed in duplicate on three samples from each developmental stage (25–45 daf, mature). Error bars indicate SE. UA, uronic acids; Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

embryo development and maturation. In mature embryos, 39% of cell wall monosaccharides were derived from UDP-GlcA.

UDP-GlcA synthesis in developing embryos

To estimate the relative contributions of the MIO and NSO pathways to the synthesis of UDP-GlcA, marker enzymes for the two pathways, UDP-GlcA PPase and UDP-Glc DH, respectively, were measured 25, 35 and 45 daf in desalted embryo extracts (Fig. 4). UDP-Glc DH activity increased 3.5-fold during the period 25–45 daf, when seed fresh weight increased approximately three-fold. Spectrophotometric assays of UDP-Glc DH activity in desalted extracts were conducted in the presence of pyrazole to prevent overestimation of enzyme activity due to the presence of alcohol dehydrogenase. Previous research has indicated that the contribution of alcohol dehydrogenase to NAD^+ reduction in the spectrophotometric UDP-Glc DH assay is significant in developing soybean embryos (Litterer et al. 2005). UDP-GlcA PPase activity increased 3.9-fold during embryo development (25–45 daf). The activity of both UDP-Glc DH and UDP-GlcA PPase increased in proportion to the increase in seed fresh weight during embryo development, but UDP-GlcA PPase activity was 35–50-fold higher than UDP-Glc DH activity.

Identification and cloning of soybean *USP1*

The gene encoding a substrate-specific UDP-GlcA PPase (EC 2.7.7.44) has not been identified. The only gene product known to exhibit activity with GlcA-1-P is

Fig. 2. Composition of developing soybean embryos. Developing embryos (cotyledons and embryonic axis) were harvested at 25, 35, 45 days after flowering (daf) and at maturity. Data points are means ± SE for duplicate determinations on three samples at each stage. Where not shown, SE bars are smaller than the symbol. CWP, cell wall polysaccharides.
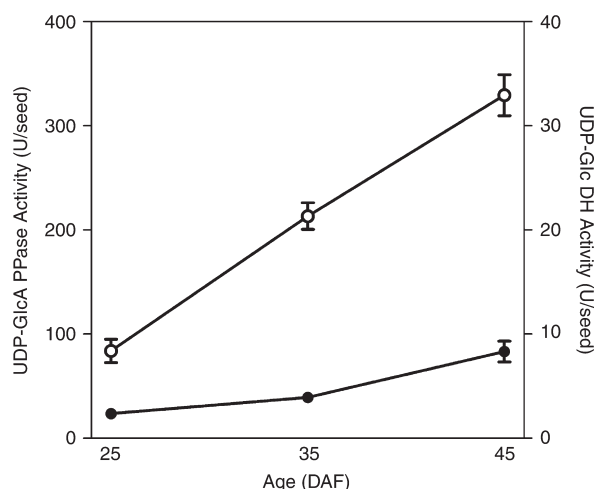
Fig. 4. Activity of UDP-glucose dehydrogenase (-●-) and UDP-GlcA pyrophosphorylase (-○-) measured in desalted extracts of developing soybean embryos harvested at 25, 35 and 45 daf. UDP-GlcA pyrophosphorylase activity was measured as the reverse reaction (UDP-GlcA → GlcA-1-P). One unit of activity is defined as μmol substrate converted min^{-1}. Where not shown, SE bars are smaller than the symbol. Data points represent mean ± SE (n = 3).

a broad-substrate USP that was recently purified from pea (Kotake et al. 2004). *Arabidopsis* has a single *USP* gene (At5g52560 or GenBank accession no. NM_124635) (Litterer, unpublished data). Soybean, a diploid resulting from an ancient tetraploidization (Hymowitz 2004), would be expected to have at least two *USP* genes. A search of the soybean expressed sequence tag database at TIGR (URL: http://www.tigr.org/tdb/tgi, accessed February 2005) with the pea sequence indicated three soybean singleton ESTs and two contigs with 85–87% identity to pea. Because none of these sequences extended to the 5′-end of the pea cDNA, 5′-RACE and RT-PCR were performed to isolate a full-length *USP* cDNA (*USP1*) (GenBank accession no. DQ267699). Sequence comparisons of full-length *USP* cDNA that we cloned with the contigs predicted from the EST search indicate that there are at least two expressed *USP* genes in soybean.

A comparison of the predicted peptide sequences of *USP* homologs from five plant species indicated that the gene is highly conserved (Fig. 5). Soybean *USP* had 92.2% identity and 96.1% similarity to pea *USP*. For the three legumes examined, identity was 87.6%. The legume peptides differed from the predicted *Arabidopsis* and rice peptides by a deletion of 12–23 amino acids near the N-terminus and a deletion of three amino acids in a short variable region between residues 190 and 200. The majority of the sequence variation among the species examined occurred in a 100 amino acid region at the C-terminus of the peptide. In *Arabidopsis*, the gene

that exhibits the highest similarity with *USP* is a putative UDP-N-acetylglucosamine pyrophosphorylase that has 26% identity and 42% similarity. No ESTs or genomic sequences homologous to *USP* were detected in fungi, metazoa, bacteria or archea, indicating that this gene is unique to plants.

Characterization of soybean *USP1*

To functionally annotate the putative soybean *USP1*, the coding sequence was cloned into an expression vector and the resulting peptide was purified from *E. coli*. The His-tag affinity-purified and thrombin-cleaved USP1 had a molecular mass of 71 kDa as determined by SDS-PAGE (data not shown). The enzyme showed high activity with GlcA-1-P, Glc-1-P or galactose 1-phosphate (Gal-1-P) as substrate, but very low activity with mannose 1-phosphate (Man-1-P), GlcNAc-1-P or glucose 6-phosphate (Glc-6-P) (Table 1). When assayed with GlcA-1-P as substrate, soybean USP1 exhibited a broad pH optimum between pH 7.5 and 9.0 (Fig. 6). Kinetic parameters for the recombinant enzyme were determined for the formation of UDP-sugars using GlcA-1-P or Glc-1-P as substrate (Table 2). For both sugar-phosphate substrates, V_{max} and apparent K_m values for UTP were similar. However, soybean USP1 exhibited higher affinity for GlcA-1-P than for Glc-1-P.

Because of the limited commercial availability of phosphorylated monosaccharide sugars to measure the forward reaction, a competition study with nucleotide sugars was conducted for the reverse reaction. The relative affinity of soybean USP1 for nucleotide sugars was evaluated by measuring their ability to competitively inhibit the reverse reaction measured with either UDP-GlcA or UDP-Glc as substrate (Table 3). If two nucleotide sugars are equally effective as substrates for the reverse reaction, each would be expected to reduce activity for the other by approximately 50%. When the reverse reaction with UDP-GlcA as substrate was measured, added UDP-Gal and UDP-Glc reduced the specific activity by 13% and 11%, respectively. Added UDP-arabinose (UDP-Ara) resulted in slight inhibition, while UDP-Xyl had little to no effect on the reaction with UDP-GlcA as substrate. Compared to the results of competition studies with UDP-GlcA as substrate, all UDP-sugars tested were more effective inhibitors of the pyrophosphorylase reaction with UDP-Glc as substrate. UDP-GlcA was the most effective inhibitor, resulting in 74% inhibition of the reaction. UDP-Gal was also effective, resulting in 37% inhibition. Added UDP-Xyl or UDP-Ara inhibited the reverse reaction only slightly. The results demonstrate the high affinity of soybean USP for
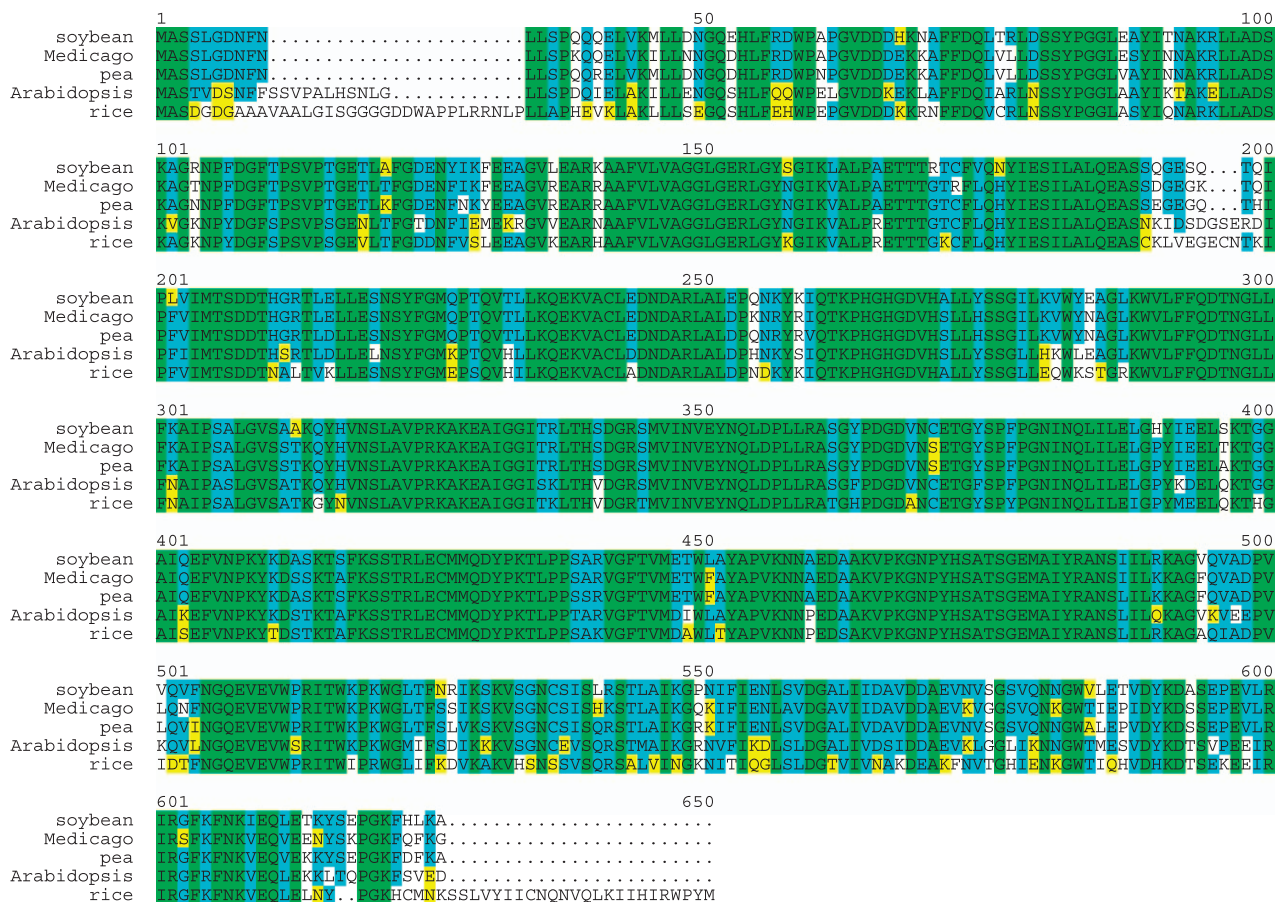
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              1                          50                                          100
soybean      MASSLGDNFN.......................LLSPQQQELVKMLLDNGQEHLFRDWPAPGVDDDHKNAFFDQLTRLDSSYPGGIEAYITNAKRLLADS
Medicago     MASSLGDNFN.......................LLSPKQQELVKILLNNGQDHLFRDWPAPGVDDDEKKAFFDQLVLLDSSYPGGLESYINNAKRLLADS
pea          MASSLGDNFN.......................LLSPQQRELVKMLLDNGQDHLFRDWPNPGVDDDEKKAFFDQLVLLDSSYPGGLVAYINNAKRLLADS
Arabidopsis  MASTVDSNFFSSVPALHSNLG............LLSPDQIELAKILLENGQSHLFQQWPELGVDDKEKLAFFDQIARLNSSYPGGLAAYIKTAKELLADS
rice         MASDGDGAAAVAALGISGGGGDDWAPPLRRNLPLLAPHEVKLAKLLLSEGQSHLFEHWPEPGVDDDKKRNFFDQVCRLNSSYPGGIASYLQNARKLLADS

              101                        150                                         200
soybean      KAGRNPFDGFTPSVPTGETLAFGDENYIKFFEAGVLEARKAAFVLVAGGLGERLGYSGIKLALPAETTTRTCFVQNYIESILALQEASSQGESQ...TQI
Medicago     KAGTNPFDGFTPSVPTGETLTFGDENFIKFFEAGVREARKAAFVLVAGGLGERLGYNGIKVALPAETTTGTRFLQHYIESILALQEASSDGEGK...TQI
pea          KAGNNPFDGFTPSVPTGETLKFGDENFNKYFEAGVREARRAAFVLVAGGLGERLGYNGIKVALPAETTTGTCFLQHYIESILALQEASSEGEGQ...THI
Arabidopsis  KVGKNPYDGFSPSVPSGENLTFGTDNFIEMPKRGVVEARNAAFVLVAGGLGERLGYNGIKVALPRETTTGTCFLQHYIESILALQEASNKIDSDGSERDI
rice         KAGKNPFDGFSPSVPSGEVLTFGDDNFVSLEAGVKEARHAAFVLVAGGLGERLGYKGIKVALPRETTTGKCFLQHYIESILALQEASCKLVEGECNTKI

              201                        250                                         300
soybean      PLVIMTSDDTHGRTLELLESNSYFGMQPTQVTLLKQEKVACLEDNDARLALEPQNKYKIQTKPHGHGDVHALLYSSGILKVWYEAGLKWVLFFQDTNGLL
Medicago     PFVIMTSDDTHGRTLELLESNSYFGMQPTQVTLLKQEKVACLEDNDARLALDPKNRYRIQTKPHGHGDVHSLLHSSGILKVWYNAGLKWVLFFQDTNGLL
pea          PFVIMTSDDTHGRTLDLLESNSYFGMQPTQVTLLKQEKVACLEDNDARLALDPQNRYRVQTKPHGHGDVHSLLHSSGILKVWYNAGLKWVLFFQDTNGLL
Arabidopsis  PFIIMTSDDTHSRTLDLLELNSYFGNKPTQVHLLKQEKVACLDDNDARLALDPHNKYSIQTKPHGHGDVHSLLYSSGLLHKVLEAGLKWVLFFQDTNGLL
rice         PFVIMTSDDTNALTVKLLESNSYFGNEPSQVHILKQEKVACLADNDARLALDPNDKYKIQTKPHGHGDVHALLYSSGLLEQWKSTGRKWVLFFQDTNGLL

              301                        350                                         400
soybean      FKAIPSALGVSAAKQYHVNSLAVPRKAKEAIGGITRLTHSDGRSMVINVEYNQLDPLLRASGYPDGDVNCETGYSPFPGNINQLILELGHYIEELSKTGG
Medicago     FKAIPSALGVSSTKQYHVNSLAVPRKAKEAIGGITRLTHSDGRSMVINVEYNQLDPLLRASGYPDGDVNSETGYSPFPGNINQLILELGPYIEELTKTGG
pea          FKAIPSALGVSSTKQYHVNSLAVPRKAKEAIGGITRLTHSDGRSMVINVEYNQLDPLLRASGYPDGDVNSETGYSPFPGNINQLILELGPYIEELAKTGG
Arabidopsis  FNAIPASLGVSATKQYHVNSLAVPRKAKEAIGGISKLTHVDGRSMVINVEYNQLDPLLRASGFPDGDVNCETGFSPFPGNINQLILELGPYKDELQKTGG
rice         FNAIPSALGVSATKGYNVNSLAVPRKAKEAIGGITKLTHVDGRTMVINVEYNQLDPLLRATGHPDGDANCETGYSPYPGNINQLILEIGPYMEELQKTHG

              401                        450                                         500
soybean      AIQEFVNPKYKDASKTSFKSSTRLECMMQDYPKTLPPSARVGFTVMETWLAYAPVKNNAEDAAKVPKGNPYHSATSGEMAIYRANSIILRKAGVQVADPV
Medicago     AIQEFVNPKYKDSSKTAFKSSTRLECMMQDYPKTLPPSARVGFTVMETWFAYAPVKNNAEDAAKVPKGNPYHSATSGEMAIYRANSIILKKAGFQVADPV
pea          AIQEFVNPKYKDASKTSFKSSTRLECMMQDYPKTLPPSSRVGFTVMETWFAYAPVKNNAEDAAKVPKGNPYHSATSGEMAIYRANSLILKKAGFQVADPV
Arabidopsis  AIKEFVNPKYKDSTKTAFKSSTRLECMMQDYPKTLPPTARVGFTVMDIWLAYAPVKNNPEDAAKVPKGNPYHSATSGEMAIYRANSLILQKAGVKVEEPV
rice         AISEFVNPKYTDSTKTAFKSSTRLECMMQDYPKTLPPSAKVGFTVMDAWLTYAPVKNNPEDSAKVPKGNPYHSATSGEMAIYRANSLILRKAGAQIADPV

              501                        550                                         600
soybean      VQVFNGQEVEVWPRITWKPKWGLTHNRIKSKVSGNCSISLRSTLAIKGPNIFIENLSVDGALIIDAVDDAEVNVSGSVQNNGWVLETVDYKDASEPEVLR
Medicago     LQNFNGQEVEVWPRITWKPKWGLTFSSIKSKVSGNCSISHKSTLAIKGQKIFIENLAVDGAVIIDAVDDAEVKVGGSVQNKGWTIEPIDYKDSSEPEVLR
pea          LQVINGQEVEVWPRITWKPKWGLTFSLVKSKVSGNCSISQRTLAIKGRKIFIENLSVDGALIVDAVDDAEVNVSGSVQNNGWALEPVDYKDTSEPEVLR
Arabidopsis  KQVLNGQEVEVWSRITWKPKWGMIFSDIKKKVSGNCEVSQRSTMAIKGRNVFIKDLSLDGALIVDSIDDAEVKLGSLIKNNGWTMESVDYKDTSVPEEIR
rice         IDTFNGQEVEVWPRITWIPRWGLIFKDVKAKVHSNSSVSQRSALVINGKNITIQGLSLDGTVIVNAKDEAKFNVTGHIENKGWTIQHVDHKDTSEKEEIR

              601                        650
soybean      IRGFKFNKIEQLETKYSEPGKFHLKA.......................
Medicago     IRSFKFNKVEQVEENYSKPGKFQFKG.......................
pea          IRGFKFNKVEQVEKKYSEPGKFDFKA.......................
Arabidopsis  IRGFRFNKVEQLEKKLIQPGKFSVED.......................
rice         IRGFKFNKVEQLELNY..PGKHCMNKSSLVYIICNQNVQLKIIHIRWPYM
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Fig. 5. Predicted peptide comparison of plant UDP-sugar pyrophosphorylases. Nucleotide sequences were translated and aligned using Vector NTI version 5.1. Soybean, GenBank no. DQ267699; Medicago, *Medicago truncatula* EST contig TC77530; pea, GenBank no. AB178642; *Arabidopsis*, locus At5g52560; rice, EST contig TC277270. Amino acids highlighted in green are strictly conserved, those highlighted in blue are highly conserved, and those highlighted in yellow are weakly similar between peptides. The legume peptides differed from the predicted *Arabidopsis* and rice peptides by a deletion of 12–23 amino acids near the N-terminus and a deletion of three amino acids in a short variable region between residues 190 and 200. The *Medicago truncatula* sequence was edited to remove a frame shift at nucleotide 1021.

UDP-GlcA as substrate in the reverse reaction (pyrophosphorolysis) compared with UDP-Glc.

Expression of *UGD* and *USP*

Tenhaken and Thulke (1996) reported that soybean had a single UDP-Glc DH (*UGD*) gene. However, analysis of expressed sequences (URL: http://www.tigr.org/tdb/tgi, accessed August 2005) in the current soybean gene database indicated that soybean has at least two highly conserved *UGD* genes. To measure expression of the *UGD* gene family, primers were designed to amplify conserved regions of the gene. Primers were also selected from conserved regions of the *USP* gene family. The relative expression levels of *UGD* and *USP* were determined by semiquantitative RT-PCR and normalized to 18S rRNA levels (Fig. 7). *UGD* transcript was more abundant than *USP* transcript in developing embryos. When normalized to 18S RNA transcript levels, the relative levels of expression for both genes exhibited only minor changes during embryo development. Both *USP*

Table 1. Activity of soybean USP with sugar-phosphate substrates. Activity of recombinant soybean USP was determined with 1 mM indicated substrate, 1 mM UTP, and 50 ng of enzyme in reaction buffer. Specific activity with GlcA-1-P, 97.0 ± 2.6 μmol min^{-1} mg^{-1}, was normalized to 100%. Results are means $\pm$ SE for triplicate experiments.

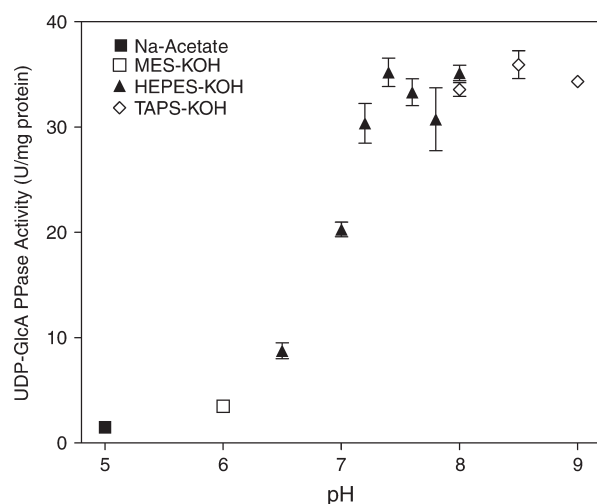| Substrate	Relative activity
GlcA-1-P	100
Glc-1-P	113.9 ± 1.7
Gal-1-P	116.4 ± 2.0
Man-1-P	1.97 ± 0.90
GlcNAc-1-P	3.57 ± 0.81
Glc-6-P	2.29 ± 1.25

Fig. 6. Effect of pH on UDP-GlcA PPase activity of recombinant USP. Specific activity (μmol min^{-1} mg^{-1}) of the reaction producing UDP-GlcA was determined in reactions containing 100 mM indicated buffer, 1 mM GlcA-1-P, 1 mM UTP and 2 mM MgCl$_2$. Data are means $\pm$ SE of triplicate experiments.

and *UGD* were also expressed in root tips, young leaves and flowers of soybean. As found in developing embryos, the transcript levels were greater for *UGD* than for *USP*. Overall, the results indicate that both gene families are widely expressed in soybean.

Discussion

During the rapid growth phase of soybean embryo development (25–45 daf), CWPs increased in parallel with oil and protein. On a weight basis, approximately 39% of CWPs in mature embryos are derived from UDP-GlcA. These results indicate that significant rates of UDP-GlcA synthesis are occurring during embryo development. Our analysis, based on marker enzyme activities (UDP-GlcA PPase, UDP-Glc DH) and transcript levels (*UGD*, *USP*), suggest that both pathways for the synthesis of UDP-GlcA are present in developing embryos and may be contributing to matrix polysaccharide biosynthesis.

In earlier research, one approach that has been used to ascertain the relative importance of the MIO and NSO

Table 2. Kinetic parameters for soybean USP with GlcA-1-P and Glc-1-P. Activity of recombinant soybean USP was determined at five concentrations of each substrate. Michaelis constants were determined by non-linear curve fitting of the means from triplicate experiments. $K_A = K_m$ value measured with substrate A; $K_B = K_m$ value measured with substrate B.

Substrate A	Substrate B	V_{max} (nmol min^{-1})	K_A (mM)	K_B (mM)
GlcA-1-P	UTP	5.03 $\pm$ 0.33	0.14 $\pm$ 0.02	0.15 $\pm$ 0.01
Glc-1-P	UTP	4.99 $\pm$ 0.27	0.23 $\pm$ 0.02	0.19 $\pm$ 0.01

Table 3. Effects of various UDP-sugars as competing substrates on UDP-GlcA and UDP-Glc pyrophosphorylase activity measured with soybean USP. Activity was measured in the reverse direction in a reaction containing 1 mM ^{14}C-labeled UDP-sugar (UDP-GlcA or UDP-Glc), 5 mM sodium pyrophosphate and 1 mM indicated competing substrate. Specific activities in the absence of competing substrate were 44.9 $\pm$ 1.5 μmol min^{-1} mg^{-1} for UDP-GlcA and 41.8 $\pm$ 1.9 μmol min^{-1} mg^{-1} for UDP-Glc.

Added competing substrate	UDP-GlcA pyrophosphorylase activity (%)	UDP-Glc pyrophosphorylase activity (%)
None	100 $\pm$ 7.8	100 $\pm$ 4.6
UDP-Glc	89 $\pm$ 3.2	–
UDP-GlcA	–	26 $\pm$ 6.7
UDP-Gal	87 $\pm$ 10.1	63 $\pm$ 7.7
UDP-Xyl	109 $\pm$ 7.3	88 $\pm$ 7.0
UDP-Ara	96 $\pm$ 2.7	89 $\pm$ 5.4

pathways for the production of UDP-GlcA in various tissues has involved measuring the activities of UDP-GlcA PPase and UDP-Glc DH (Roberts 1971, Roberts and Cetorelli 1973, Amino et al. 1985, Witt 1992). These enzymes are considered to be marker enzymes for the MIO and NSO pathways, respectively. Our results comparing the activities of these marker enzymes in developing soybean embryos showed that UDP-GlcA PPase activity was 35–50-fold greater than UDP-Glc DH activity. Significant differences in the relative activities have been observed in other tisssues. For example, high UDP-GlcA PPase activity, but low UDP-Glc DH activity, was measured in barley seedlings (Roberts 1971). These
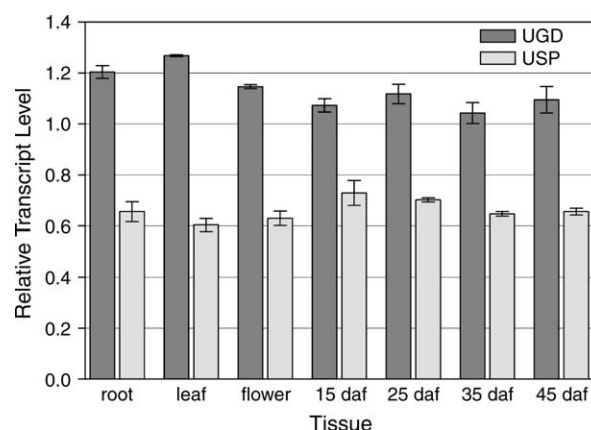

Fig. 7. Relative transcript levels of *UGD* and *USP* quantified by competitive RT-PCR. 18S rRNA, *UGD* and *USP* transcripts were amplified in the same reaction and normalized to 18S rRNA transcript levels. Data are the mean $\pm$ SE of three independent RNA samples from each tissue. Roots, 2-cm root tips of seedlings; leaves, rapidly expanding leaves 1–2 cm in length; 15–45 daf, embryos at 15–45 days after flowering.

results were interpreted to suggest that the MIO pathway was predominant in barley seedlings (Roberts 1971). However, our results and those of other studies where marker enzymes for the NSO and MIO pathways were compared need to be interpreted with caution. Enzyme activity measured in vitro may not provide an accurate measurement of in vivo activity. For example, the concentrations of substrates and possible feedback regulators in vivo are unknown. In addition, for the MIO pathway, UDP-GlcA PPase is only one of three enzymes in the pathway (Fig. 1), and its activity in vivo may not be limiting flux through the pathway.

The gene for UDP-GlcA PPase (EC 2.7.7.44) has not been identified. However, *USP*, a gene that encodes a broad-substrate pyrophosphorylase that exhibits activity with GlcA-1-P, may function as UDP-GlcA PPase. *USP* was recently cloned in pea (*Pisum sativum*) (Kotake et al. 2004). We cloned and characterized a soybean homolog of pea *USP*. Our results verified that the soybean gene encodes a broad-substrate USP with properties similar to those of pea USP. Both enzymes had a molecular mass of approximately 70 kDa and exhibited broad substrate specificity, with high activity observed with Gal-1-P, Glc-1-P and GlcA-1-P. Furthermore, both exhibited a similar temperature optimum and requirement for magnesium and UTP as cosubstrate. However, there are significant differences between the properties of the soybean and pea enzymes. Soybean USP exhibited a pH optimum in the range 7.5–9.0, whereas the optimum range for pea USP was 6.5–7.5 (Kotake et al. 2004). The pH optimum for the soybean USP is similar to the optimum for plant UDP-glucose pyrophosphorylase (Otozai et al. 1973, Sowokinos et al. 1993). Relative activities with GlcA-1-P, Glc-1-P and Gal-1-P are approximately equivalent for soybean USP. In contrast, for pea USP, the activities for these substrates exhibited a broader range (Kotake et al. 2004). The activity measured with GlcA-1-P was only 71% of that detected with Glc-1-P as substrate. Furthermore, the affinity of pea USP was greater for Glc-1-P than for GlcA-1-P, whereas the inverse was observed with soybean USP. Based on determination of kinetic constants and competition experiments conducted with UDP-sugars, our results showed that soybean USP exhibits the highest affinity for GlcA-1-P (forward reaction) and UDP-GlcA (reverse reaction).

It is difficult to evaluate whether UDP-Glc PPase activity characterized in earlier studies represents the gene product of USP. These studies were conducted with semipurified fractions, and the properties of the purified enzyme are not known (Feingold et al. 1958, Roberts 1971, Roberts and Cetorelli 1973, Dickinson et al. 1977, Hondo et al. 1983, Witt 1992). In previous research, the two most purified enzyme fractions exhibiting UDP-GlcA

PPase activity were from common cattail pollen (Hondo et al. 1983) and barley seedlings (Roberts 1971). In common cattail pollen, the protein exhibiting UDP-GlcA PPase activity was purified 600-fold and had properties similar to those of soybean USP. The enzyme from common cattail had a molecular mass of approximately 71 kDa, a requirement for magnesium, and a K_m value for GlcA-1-P similar to that of soybean USP. However, the cross-reactivity of the common cattail enzyme for other monosaccharide 1-phosphate sugars was not examined. The barley UDP-GlcA PPase fraction was purified approximately 80-fold. The enzyme required Mg-UTP as cosubstrate and, like soybean USP, exhibited a pH optimum in the range pH 8–9. However, in contrast to soybean USP, the barley enzyme had an estimated molecular mass of 35 kDa and exhibited no cross-reactivity with Glc-1-P. Although our results clearly indicate high levels of UDP-GlcA PPase activity in developing embryos, it is not clear to what extent the enzyme activity measured in desalted extracts of soybean represents the activity of USP. It is possible that other pyrophosphorylases capable of converting UDP-GlcA to GlcA-1-P are present in the desalted extracts from soybean embryos.

Our results suggest that USP plays an important role in developing soybean embryos. *USP* transcript is present in developing soybean embryos. Furthermore, high levels of UDP-GlcA PPase activity are found in developing embryos, and the gene product of USP exhibits high activity with GlcA-1-P. However, the specific role that *USP* plays in developing soybean embryos is not clear. The broad substrate specificity of USP suggests an important role in the salvage pathway for the synthesis of nucleotide sugars (Kotake et al. 2004). In this pathway, monosaccharides that are hydrolyzed from polysaccharides, glycolipids and glycoproteins are recycled back into the nucleotide sugar pool (Feingold and Avigad 1980, Carpita and McCann 2000). This function may be important during cell wall growth and modification. Pulse chase studies of CWPs indicated significant recycling of Ara and Gal (Gibeaut and Carpita 1991, Gorshkova et al. 1997, Lozovaya et al. 1996). However, it is not clear how important nucleotide salvage and recycling are in the synthesis and turnover of CWPs in developing soybean embryos.

It is also possible that soybean USP functions as the terminal enzyme of the MIO pathway in developing embryos. If this is the case, USP would play an important role in the synthesis of matrix CWPs in the developing embryo. The relatively high activity and affinity of soybean USP for GlcA-1-P suggests that it could play this role. However, the contribution of the *USP* gene product to UDP-GlcA PPase activity measured in developing embryo extracts is uncertain. Further research is needed

to define the role of USP in cell wall metabolism, including its involvement in recycling sugars during cell wall growth and a possible role as the terminal enzyme in the MIO pathway.

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